

Enzymatic activity of soluble phospholipase A₂ does not affect the specific IgE, IgG4 and cytokine responses in bee sting allergy

D. WYMAN, C. A. AKDIS, T. BLESKEN, M. AKDIS, R. CRAMERI and K. BLASER

Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

Summary

Background The soluble bee venom phospholipase A₂ (PLA) represents the major allergen/antigen for allergic and hyperimmune individuals following bee sting. A number of studies implicate enzymes, and PLA in particular, as potent allergens. We have studied specific activation of T cells by enzymatically active and inactive mutants of PLA, and secretion of cytokines regulating IgE and IgG4 antibody formation.

Methods Recombinant (r) wild type PLA (rPLA-WT) and an enzymatically inactive rPLA (rPLA-H34Q) were produced in *Escherichia coli*. Eleven bee venom allergic patients and three hyperimmune, healthy individuals were included in the study. After specific stimulation of PBMC with the rPLA variants, proliferative response, IFN γ , IL-2, IL-4, IL-5, and IL-13 production, as well as total and PLA-specific IgE and IgG4 production, were analysed.

Results Similar levels of specific B cell recognition, proliferative and cytokine responses were observed after stimulation with either enzymatically active or inactive rPLA. In addition, equal amounts of antigen-specific and total IgE and IgG4 antibodies were produced by stimulation with both forms of rPLA.

Conclusions The enzymatic activity of PLA does not influence the specific activation and cytokine production by T cells from bee venom-sensitized or hyperimmune individuals, or the IgE/IgG4 antibodies synthesis by B cells *in vitro*.

Keywords: allergen, bee venom allergy, enzymatic activity, cytokines, IgE-regulation

Clinical and Experimental Allergy, Vol. 28, pp. 839–849. Submitted 20 October 1997; revised 14 January 1998; accepted 21 February 1998.

Introduction

Allergic diseases are clinical manifestations of excessive IgE antibody (Ab) responses occurring in some individuals following exposure to environmental antigens. In addition, classical allergens are also strong inducers of IgG4 Abs [1,2], reflecting normal immunity to the antigen. Allergen-specific Th cells from atopic individuals generally display TH₂ type cytokine patterns with only little IFN γ production [3]. The Th₂ cytokines IL-4 and IL-13 are potent inducers of IgE synthesis [4,5], while IFN γ acts as an IgE inhibitor [3,5]. In contrast, IgG4 Ab production by memory B cells

and normal immunity to allergens depends on IL-2 and IFN γ secretion [6,7].

A number of studies have suggested that enzymes in particular appear to be potently allergenic [8–14]. Besides the soluble PLA, acid phosphatase and hyaluronidase are known allergens in bee venom allergy [15]. Furthermore, several of the house dust mite allergens are known to be enzymes [16–20]. For instance, Der p 1 shows sequence homology to enzymes from the cysteine protease family [16]. Moreover, papain, another cysteine protease, was found to induce asthma in exposed subjects [21]. Various enzymes, such as metalloprotease, Mn-superoxidedismutase, aspartic proteinase, and peptidyl-prolyl *cis/trans* isomerase were demonstrated to be allergens of the mould *Aspergillus fumigatus* [22,23]. In addition, it was shown that

Correspondence: Dr C. A. Akdis, SIAF, Obere Strasse 22, CH-7270 Davos Platz, Switzerland.

a proteinase from *Aspergillus fumigatus* directly induces human epithelial cell detachment [24]. It was also demonstrated that the acid protease secreted by *Candida albicans* is an important allergen in *C. alb*-related mucosal allergy [25]. Up to now, it has been unclear why this category of proteins especially provokes allergic immune responses, and whether the enzymatic activity and specific function of the enzymes play a role in the development of allergy.

The soluble PLA is the major allergen and immunogen in bee venom-sensitized individuals [26,27]. Generally, bee stings elicit anti-PLA Abs that recognize conformational, discontinuous epitopes [28]. In a normal immune response, low affinity IgG1 anti-PLA Abs are initially detected [1,2]. High-affinity IgG4 anti-PLA Abs are generated after repeated exposure [2,29], or by prolonged specific immunotherapy of allergic patients [30,31]. In contrast, individuals allergic to bee sting develop IgE anti-PLA Abs [26,29–31]. PLA is a biochemically well-defined single chain glycoprotein of 14–16 kDa, consisting of 134 amino acids [32,33] with a single carbohydrate side chain at position Asn13. The protein contains five intrachain disulphide bridges, as shown in the 3D-structure, resolved by X-ray crystallography [33]. Besides a calcium ion and its binding loop, the three amino acids His34, Asp64, and Tyr87 are involved in the catalytic mechanism of PLA [34]. The His residue at position 34 of PLA is crucial for a functional catalytic centre. The hydrolytic activity toward the 2-acyl group of *L*-1,2 diacylphosphatides is directed preferentially against substrate aggregates, such as cell membranes, micelles, monolayers, or vesicles. Moreover, an important biological function of PLA consists of release of arachidonic acid from phospholipids. This fatty acid plays a key role in cellular regulation as an intercellular and intracellular messenger, but is absent as a free fatty acid from most tissues and cells [35]. PLA was also found at inflammatory sites, such as peritoneal exudates and in human rheumatoid arthritis [36]. Therefore, it could be suspected that PLA affecting cell membranes, and cell signalling in particular, can display direct effects on immunoregulation.

Mast cell degranulation can be induced by basic molecules [37], and PLA (pI = 7.76) was shown to express a degranulatory activity on mast cells [38–40]. In the present study, we investigated the binding capacity of specific and nonspecific B cells, T cell activation, cytokine production, and their regulatory effects on IgE and IgG4 synthesis, in response to rPLA-WT or the enzymatically inactive rPLA-H34Q. We demonstrate that rPLA-H34Q binds to antigen presenting cells (APC) in equal amounts, compared to the wild type form. Moreover, rPLA-H34Q induces similar proliferative cytokine responses and Ig isotype synthesis in PBMC from bee venom-sensitized or hyperimmune individuals. Thus, we conclude that the PLA enzymatic

activity does not influence cellular and humoral immune responses in bee sting allergy.

Materials and methods

Subjects

Eleven bee venom allergic patients (mean age: 33 years), with a history of severe systemic allergic reactions of grade III–IV [41] after a bee sting, and three hyperimmune, healthy individuals were included in the study. All allergic patients demonstrated positive intracutaneous reactions to honey bee venom (ALK, Horsholm, Denmark) at $< 10^{-4}$ g/L and bee venom specific serum IgE Abs ≥ 2 kU/L, as estimated by CAP assay (Pharmacia Diagnostics Co., Uppsala, Sweden).

Reagents

Native phospholipase A₂ (nPLA) was purchased from Boehringer AG (Mannheim, Germany). IL-2 and IL-4 were obtained from Novartis AG, Basel, Switzerland. Soluble CD40 ligand (sCD40L) was produced from the transfected cell line 8–40–1 [42], originally generated by Dr P. Lane (Institute for Immunology, Basel, Switzerland), cultured for 3 days in CG medium (Vitromex, Vilshofen, Germany). It was standardized according to maximal IgE-inducing capacity, in a 12-day culture of PBMC costimulated with 25 ng/mL IL-4 [7]. Supernatants from the corresponding untransfected cell line J558L (kindly provided by Dr M. Reth, University of Freiburg, Germany) were used as control.

Production of enzymatically active and inactive rPLA

PLA has been cloned and produced as an inclusion body protein in *E. coli*. Formation of enzymatically active rPLA-WT with full lipid binding affinity required an additional step of protein refolding *in vitro* [33,43]. The enzymatically inactive H34Q point-mutated PLA variant rPLA-H34Q, in which H34 was replaced by Gln, has been generated as described [40].

Production of rPLA-WT

A synthetic gene encoding the mature form of PLA was expressed in *E. coli*. This enzyme was produced as a N-terminal 6xHis-tagged protein, yielding between 40 and 50 mg/L of culture.

Production of rPLA-H34Q

The site-specific substitution of the His at position 34 with Gln was introduced by PCR. A gene fragment carrying the H34Q mutation was amplified using the primer 5'-ACG-CATGCTGTCGCACCCAGGACA-3' together with the flanking T7 primer (Stratagene, La Jolla, CA, USA) and the synthetic gene encoding wild type PLA in a pBluescript II SK vector as the template [43]. The PCR reactions were performed in 100 μ L, containing the appropriate buffer, 200 μ M of each deoxynucleotide triphosphate, 0.5 μ M of each 5'- and 3'-primer, 10 ng of target DNA, and 1 U of Vent DNA polymerase (New England Biolabs, Schwalbach/Taunus, Germany). The samples were subjected to two cycles of amplification, consisting of denaturing for 1 min at 95 °C, annealing for 1 min at 45 °C, and extending for 1 min at 72 °C, followed by 25 cycles under identical conditions, except that the annealing temperature was raised to 50 °C. The PCR product was purified by agarose gel electrophoresis and recovered with GeneClean (Bio 101 Inc., La Jolla, CA). This mutant gene fragment was digested with *Sph*I and *Hind*III and cloned into the same restriction sites of the wild type PLA expression construct [43]. The presence of the mutation was confirmed by DNA sequencing, and a construct carrying the correct insert was selected for protein expression.

Refolding of rPLA

The expressed proteins (50 mg) were dissolved in 7 M guanidine-HCl, 0.3 M Na₂SO₃ (pH 8.3), at a concentration of 0.5 mg/mL, and a 1/20 volume of Thannhauser reagent [44] was added to sulphonate the cysteine thiols. The solution was dialysed twice for 4 h against 3.5 L of a freshly made solution containing 2 M urea, 4 mM EDTA, 0.1 M NH₄Cl and 20 mM sodium borate at pH 8.3. Then, 56 mL of 0.5 M cysteine in water and 17.5 mL of 0.2 M cystine in 1 M HCl were added to the dialysis buffer, the pH adjusted to 8.3 with 10 M NaOH, and dialysis continued in the dark. The protein solution was decanted and dialysed against 50 mM Tris-HCl, pH 9.0 at 4 °C. The solution was loaded onto a SP-Sephadex C-25 (Pharmacia Diagnostics Co.) column, previously equilibrated with SP buffer, and the protein was eluted with a linear gradient of 50 mM to 750 mM Tris-HCl buffer (pH 9.0). Protein-containing fractions were pooled, dialysed against 5 mM acetic acid, and lyophilized.

FACS analysis

The cell binding capacity of PLA and PLA variants was determined in the PLA-specific Epstein-Barr virus (EBV) transformed human B cell line BuB1 and the PLA

non-specific EBV-B cell line BuB from the same donor [45]. The BuB1 cells produce monoclonal IgG4/ λ anti-PLA antibodies [45]. Cells ($10^5/50 \mu$ L) were incubated with FITC-labelled rPLA (0.64 FITC/PLA) [46] for 10 and 120 min. Mouse IgG1-FITC was used as control. The cells were washed with 5 mL HBSS and fixed in 2% paraformaldehyde. Fluorescence analysis was performed on an Epics Profile flow cytometer (Coulter Corp.).

Enzymatic activity and toxicity of PLA

The enzymatic activity of nPLA and rPLA-WT was determined by a spectrophotometric assay as described in [47]. Briefly, 10 ng PLA was added to different amounts of the short chain substrate diC₆-thioPMe ([racemic 2,3-Bis(hexanoylthio)propylphosphomethanol Lithium Salt], a gift from Dr M. H. Gelb, University of Washington, Seattle, USA). $V_{\max}$ was determined by using a kinetic microplate reader (Molecular Devices) and monitoring the OD at 405 nm for 8 min.

The cell toxicity of PLA was assessed by viability and proliferation assays of peripheral blood mononuclear cells (PBMC), EBV transformed B cell lines and cytotoxic lymphoid line cells. Concentrations of less than 50 μ g/mL, as used in our experiments, were non-toxic.

T cell cultures

PBMC were isolated by Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were washed and resuspended in RPMI 1640 medium supplemented as previously described [30]. The induction of PLA-specific T cell cultures and restimulation for determination of Ag-induced cytokines was performed as previously reported [30]. Briefly, 10^6 cells/mL were stimulated in a 24-well tissue culture plate (Costar Corp., Cambridge, MA, USA) with 1 μ M of PLA preparations. Cultures were expanded in medium supplemented at day 7 with a mixture of IL-2 (25 U/mL) and IL-4 (25 ng/mL). After 12 days, cells were harvested, washed three times with PBS and resuspended in 1 mL medium in a 24-well plate. Restimulation was done in duplicate with 1 μ M of the same antigen as before, in the presence of 10^6 autologous, 3000 Rad irradiated, PBMC. Supernatants were harvested 16 h later for determination of IL-2 and IL-4 and 48 h later for IL-5, IL-13 and IFN γ . T cell proliferation was determined in triplicates, by stimulating 2×10^5 PBMC in 200 μ L medium with various concentrations of PLA, in 96-well flat bottom tissue culture plates for 5 days. They were pulsed with 1 μ Ci/well of [³H]thymidine (DuPont/New England Nuclear, Boston, MA, USA), and incorporation of labelled nucleotide was determined after 20 h in a LKB beta plate reader (Wallac, Pharmacia, Turku, Finland).

Quantification of cytokines

The solid phase ELISAs for IFN γ , IL-5, and IL-13 were as described before [7,30]. Briefly, 96-well microtiter plates (Maxisorb, Nunc, Roskilde, Denmark) were coated with MoAb 43–11 to human IFN γ and developed with biotinylated MoAb 45–15 and peroxidase labelled ExtrAvidin (Sigma Chemicals Co., Buchs, Switzerland). The sensitivity of the IFN γ ELISA was < 10 pg/mL. Anti-IFN γ MoAbs and IFN γ standard were gift from Novartis AG. IL-5 was determined by using MoAb TRFK5 for coating and biotinylated MoAb JES15A10 for detection (MoAbs and IL-5 standard from PharMingen, St. Louis, MO, USA). The detection limit of the IL-5 ELISA was 50 pg/mL. For the detection of IL-13, the MoAb JES10–2F9 (DNAX, Palo Alto, CA, USA) was used for coating. Recombinant IL-13 from PeproTech Inc. (Rocky Hill, NJ, USA) was used as a standard. Rabbit anti-IL-13 (PeproTech Inc.) and alkaline phosphatase-labelled goat anti-rabbit Ab (Zymed Labs. Inc., San Francisco, USA) were used for detection. The detection limit was 300 pg/mL of IL-13. In this case, the chromogenic substrate 4-nitrophenyl-phosphate-disodium-hexahydrate (E. Merck, Darmstadt, Germany) in diethanolamine buffer, pH 9.8, was used.

IL-2 activity was measured by [^{3} H]thymidine uptake of CTLL cells, as described [48].

IL-4 was measured by coating with MoAb 8F12 and biotin-conjugated MoAb 3H4 (both from Novartis AG) and peroxidase-labelled ExtrAvidin was used for detection. The detection limit was < 100 pg/mL.

Induction of PLA-specific and total IgE and IgG4

For PLA-specific IgE and IgG4 induction, Ag-specific T cell cultures were generated by stimulation and expansion with 1 μ M of rPLA-WT or with rPLA-H34Q, in the presence of IL-2 and IL-4. At day 12, washed cells (2.5×10^5) were reconstituted together with 2.5×10^5 freshly purified autologous B cells in 500 μ L of insulin and transferrin-supplemented medium. After an additional 12 days of culture with 0.6×10^{-4} μ M of the same PLA preparation, supernatants were harvested for determination of IgE and IgG4 anti-PLA concentrations.

The IgE and IgG4 *in vitro* production was studied as described [7]. In 48-well tissue culture plates, 0.5×10^6 PBMC were stimulated with 25 ng/mL IL-4 and 25% sCD40L, in the presence of 0, 0.01, 0.1 and 1 μ M of rPLA-WT or rPLA-H34Q. The supplemented RPMI-1640 medium (0.5 mL/well) was supplied with 40 μ g/mL human transferrin and 4 μ g/mL bovine insulin (both from Sigma Chemicals Co.).

Quantification of PLA-specific and total IgE and IgG4

IgE and IgG4 anti-PLA contents in culture supernatants were measured in duplicates by ELISA [6,7,30]. PLA-coated plastic plates (Maxisorb, Nunc) were incubated with the culture supernatants. Biotinylated anti-IgE mAb 6–7 (Novartis AG) and peroxidase-labelled ExtrAvidin were used, to develop IgE anti-PLA Abs. Anti- γ 4 MoAb RJ4 (Oxoid Ltd, Basingstoke, UK) and peroxidase-labelled antimouse Ig antibody (Tago Inc., Burlingame, CA) were used in IgG4 anti-PLA ELISA. Sera from patients allergic to bee venom were calibrated with RAST (Pharmacia Diagnostics Co.) and used as IgE anti-PLA standard [7]. Human PLA-specific IgG4 MoAb BVA2 was used as IgG4 anti-PLA standard [28]. The sensitivity of these ELISAs were < 0.15 ng/mL of IgE anti-PLA and < 0.2 ng/mL of IgG4 anti-PLA.

Total IgE was determined by sandwich-ELISA, using anti- ϵ MoAb 14–41 (Novartis AG) for coating and biotinylated MoAb 6–7 for detection. The detection limit was 0.2 ng/mL of human IgE standard (Behringwerke AG, Marburg, Germany). Mouse anti- γ 4 MoAb RJ4 and peroxidase-conjugated anti-human IgG (Sigma Chemicals Co.) were used to quantify total IgG4. The detection limit of IgG4 was 0.6 ng/mL of WHO reference serum 67–97.

Results

Enzymatic activity and specific IgG binding of PLA preparations

The enzymatic activity of nPLA and rPLA was determined by a spectrophotometric assay, using the substrate diC₆-thioPMe [47]. As shown in Fig. 1(A), nPLA and refolded rPLA-WT possess similar enzymatic activity, whereas rPLA-H34Q had no catalytic activity to the substrate. Different batches of refolded rPLA-WT had a K_m of 75% to 91% of nPLA (data not shown). To control refolding of rPLA-WT and rPLA-H34Q, the human IgG4 anti-PLA MoAbs BVA1 and BVA2, recognizing a similar discontinuous epitope [28], were used. As demonstrated for BVA2 (Fig. 1B), the PLA variants possess similar binding capacity and may therefore display a similar three-dimensional structure. We also examined the binding capacity of serum IgE and IgG4 anti-PLA Abs from pooled serum of bee sting-sensitized patients. We observed that nPLA, rPLA-WT, and rPLA-H34Q bind to the same extent to serum IgE and IgG4 anti-PLA Abs, whilst the unrefolded rPLA-WT was not recognized by the specific isotypes (data not shown).

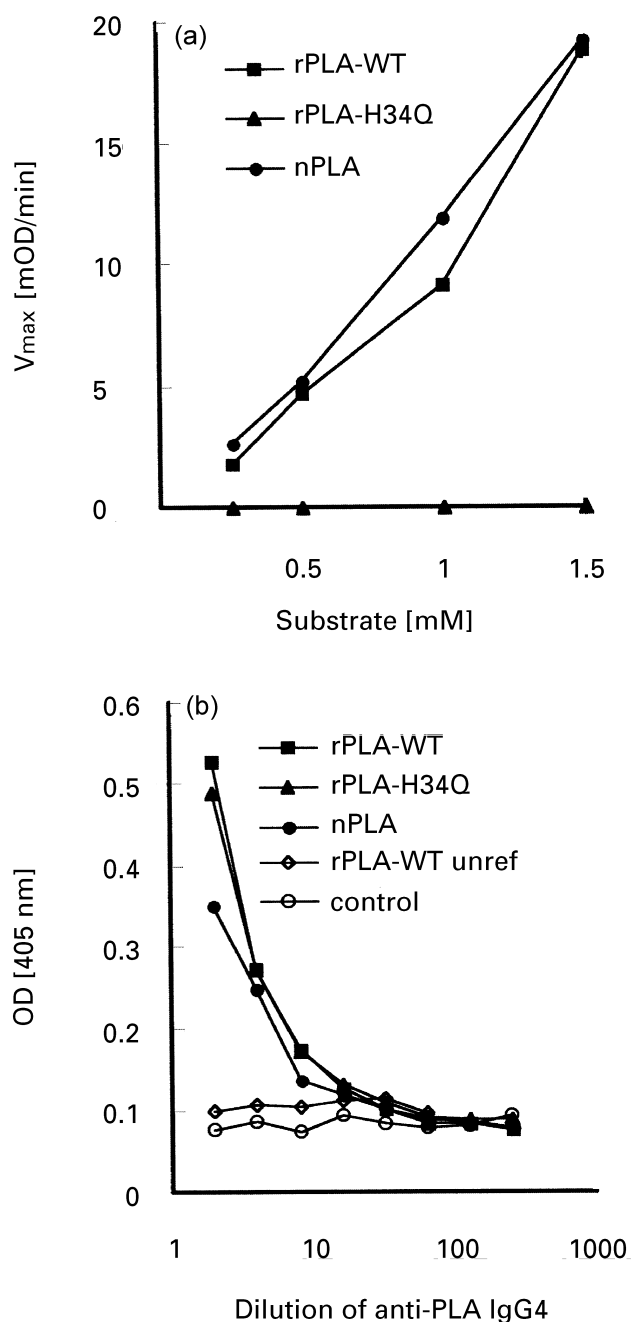


Fig. 1. Enzymatic activity and antibody binding of different PLA. The kinetics of nPLA and rPLA-WT are alike and the pointmutated rPLA-H34Q showed no enzymatic activity towards the substrate diC6-thioPMe (A). The antibody BVA2, recognizing a conformational epitope, binds to nPLA, rPLA-WT and rPLA-H34Q with a similar affinity, whereas unfolded rPLA-WT (rPLA-WT unref) shows no IgG4 Ab binding (B). The data are representative for 10 rPLA batches.

Uptake of PLA by B cells does not depend on its enzymatic activity or on B cell specificity

It was shown that Th cell clones proliferate equally well in response to the PLA presented by either specific BuB1 or non-specific BuB cells [45]. This effect was not related to different levels of MHC class II expression by the EBV-B cell lines [45]. We investigated, whether the enzymatic activity of PLA plays a role in antigen uptake by the APC. Both BuB1 and BuB cells were incubated with FITC-labelled rPLA-WT and rPLA-H34Q. After 10 min, there was significant binding of PLA compared with the control (Fig. 2). We observed an increase in mean fluorescence at 120 min, indicating that not all PLA molecules were bound to the APC within the first 10 min. PLA was not detectable after 7 h in the medium, as determined by HPLC with a C4 column (125×2 mm, 300 Å, 5 µm) that was developed with a linear acetonitrile gradient (data not shown). Enzymatic activity did not alter the APC binding of PLA, either in antigen-specific or unspecific APC.

Enzymatic activity does not influence specific T cell proliferation

In order to elucidate whether the PLA enzymatic activity may influence the specific proliferative T cell response, enzymatically active and inactive rPLA preparations were used to stimulate PBMC. In a first experiment PBMC proliferation to different doses of PLA was established measuring [^{3} H]thymidine incorporation ([^{3} H]TdR). In Fig. 3(A) it is demonstrated that both rPLA-WT and the mutated rPLA-H34Q generate similar responses and reach plateau values at 1 µM of antigen. Therefore, this concentration was used to stimulate PBMC of 11 bee venom allergic and three non-allergic, hyperimmune individuals with both PLA variants (Fig. 3B). Again, at this concentration it appeared that, independent of an enzymatic activity of the protein, there was no difference in proliferative response. The mean proliferative response ($\pm$ SEM) to 1 µM of antigen was the same with rPLA-WT (11706 ± 2569 cpm) and rPLA-H34Q (15857 ± 5389 cpm). PBMC from the three hyperimmune individuals do not respond differently, compared with the allergic patients.

Enzymatic activity of PLA does not alter cytokine production

Distinct cytokine profiles direct immune responses towards normal reaction or allergic inflammation. Figure 4 shows allergen-induced cytokine production in response to enzymatically active and inactive rPLA. PBMC from bee venom sensitized donors were specifically stimulated and expanded with either form of rPLA, in the presence of IL-2 and IL-4. Cytokines were measured after washing and restimulation

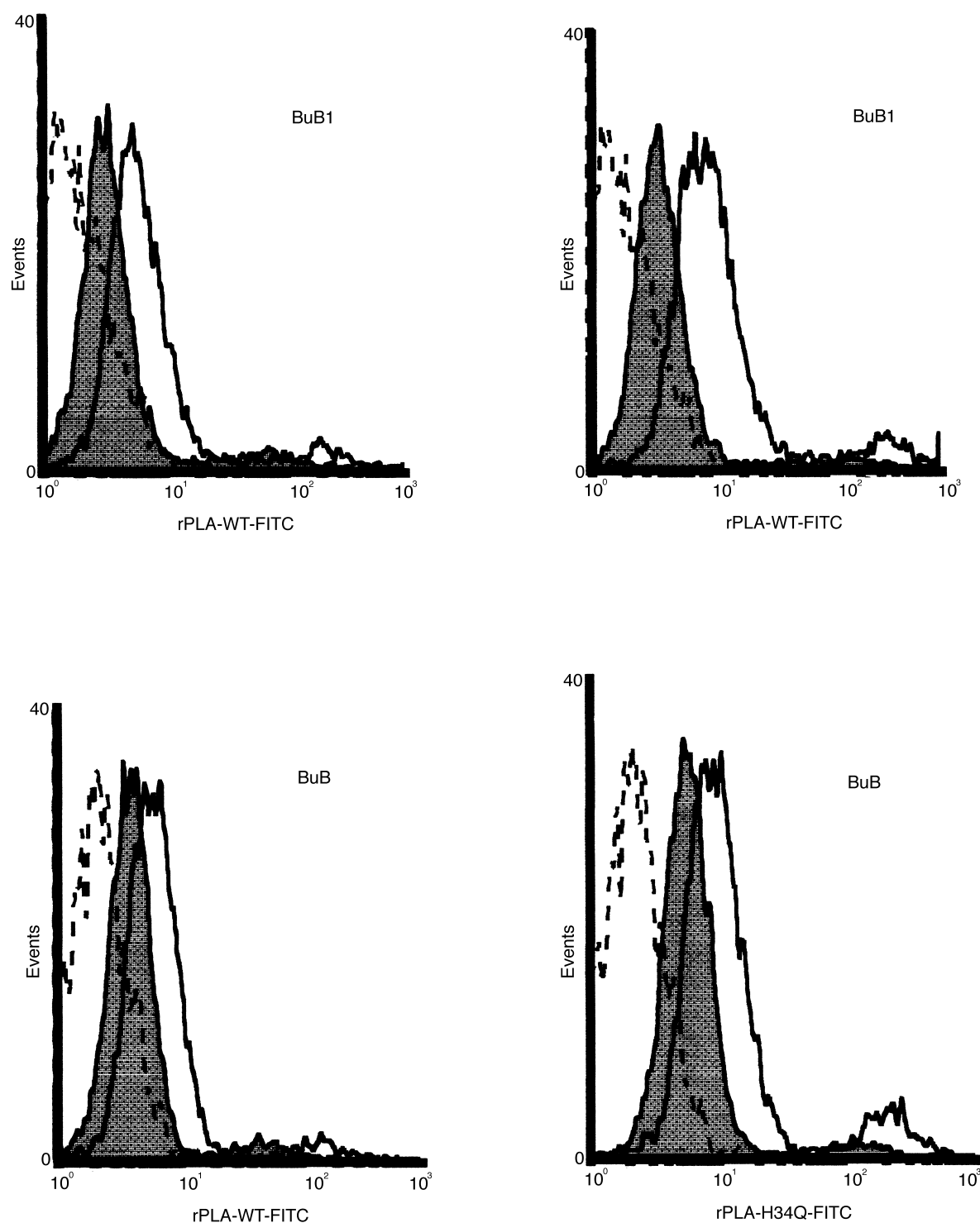


Fig. 2. Enzymatic activity of rPLA has no effect on APC binding. PLA-specific BuB1 and nonspecific BuB cells were incubated with FITC-labelled enzymatically active (rPLA-WT-FITC) or inactive (rPLA-H34Q-FITC) rPLA for 10 min (filled) and 120 min (open). FITC-labelled mouse IgG1 was used as control for 2 h (dashed line). There was no difference between the amounts of bound rPLA or the speed of binding observed for enzymatically active and inactive rPLA. Due to its high affinity for lipids, similar amounts of rPLA bound to nonspecific and to specific APC.

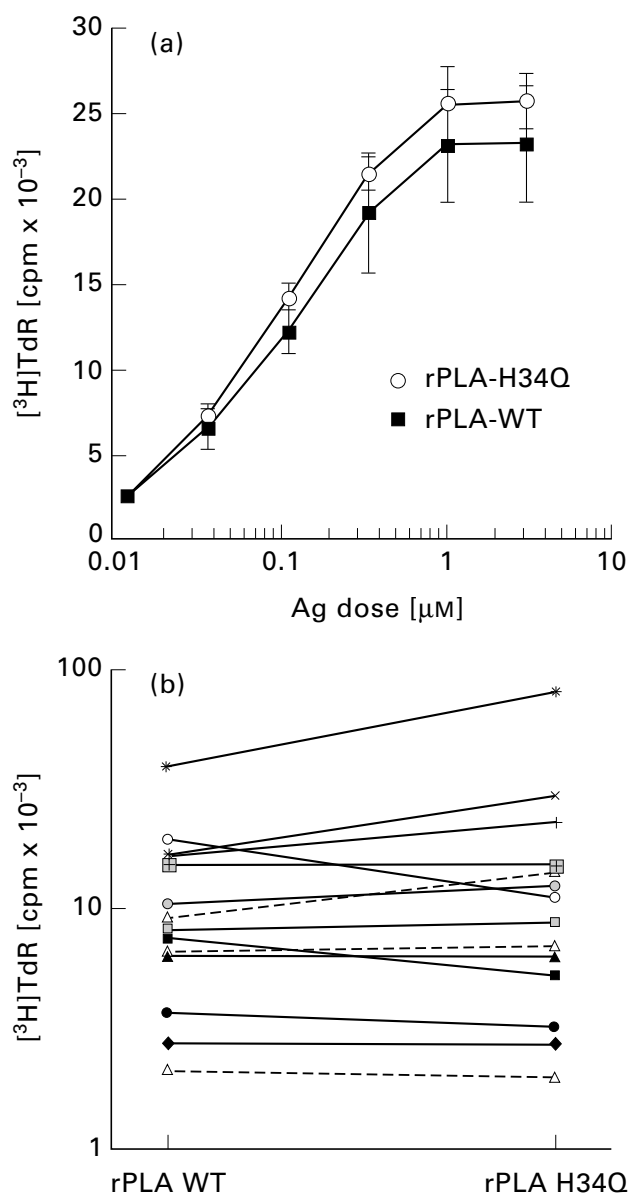


Fig. 3. Response of PBMC to rPLA-WT and to rPLA-H34Q. Both forms of PLA show same proliferative response reaching a plateau value at a concentration of 1 μM (A). Proliferative T cell response to 1 μM of the enzymatically active rPLA-WT and inactive rPLA-H34Q in 11 allergic (solid lines) and 3 hyperimmune (dashed lines) individuals. In all cases the proliferative response was not significantly altered by the PLA enzymatic activity. The proliferative response was similar for hyperimmune and allergic donors (B).

of cultures with the same PLA preparation as used for primary stimulation, in the presence of irradiated autologous PBMC. Both TH₂-type cytokines IL-4, IL-5 and IL-13, and TH₁-type cytokines IL-2 and IFN γ were secreted in equal amounts after stimulation with rPLA-WT compared with rPLA-H34Q. In addition, the ratio of IL-4:IFN γ produced

by different patients demonstrates the equality of the two antigens used. The cytokine secretion by PBMC from allergic donors was not significantly different compared with the PBMC from hyperimmune donors.

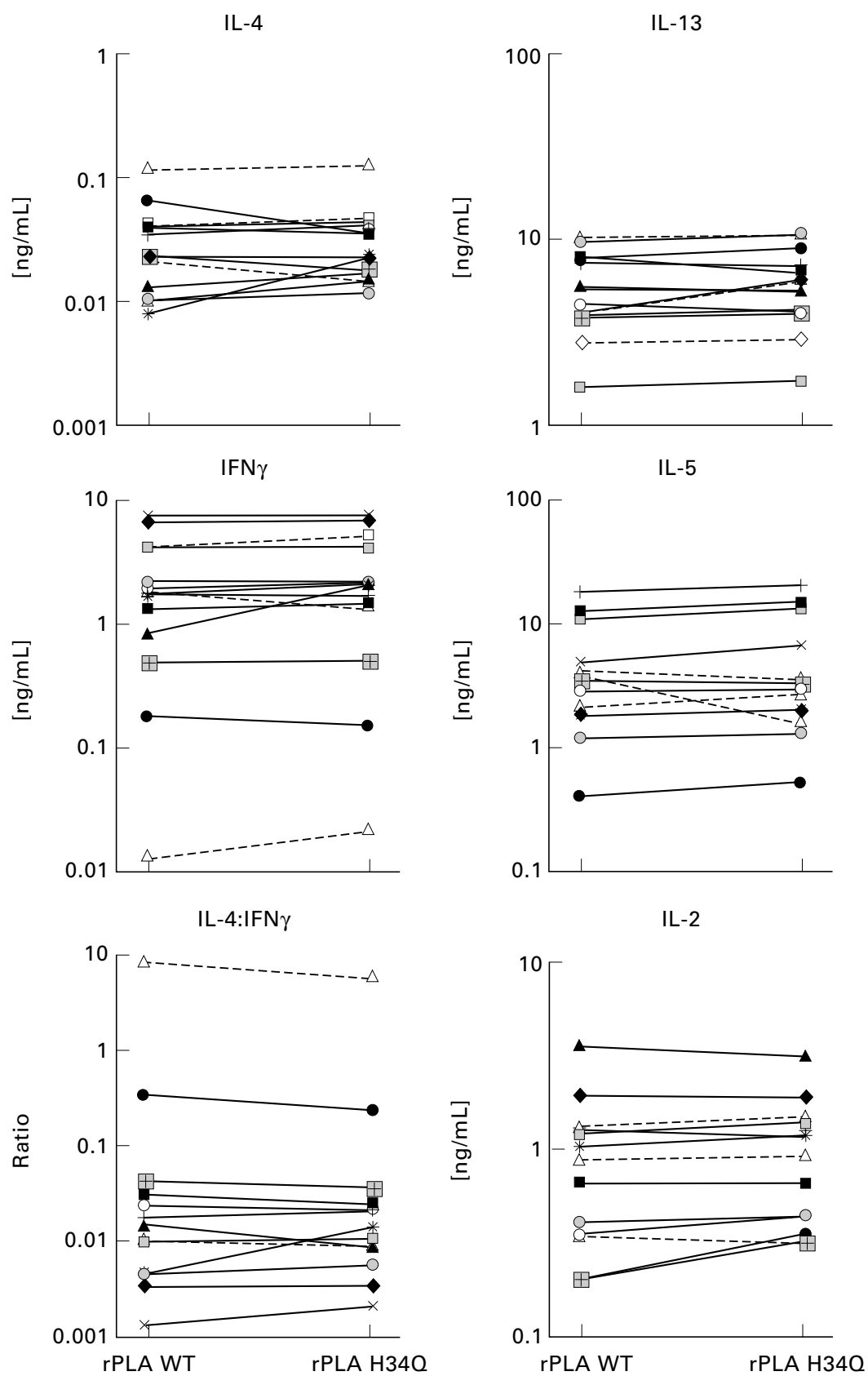
Induction of PLA-specific IgE and IgG4 Abs by rPLA-WT and rPLA-H34Q

To analyse whether enzymatic activity could play a role in Ig synthesis, PBMC were stimulated with different amounts of enzymatically active or inactive rPLA, in the presence of sCD40 L and IL-4 as described [7]. As shown in Fig. 5(A), IgE and IgG4 were produced in similar amounts after stimulation with either enzymatically active or inactive rPLA. The effect of enzymatic PLA activity on differential PLA-specific IgE and IgG4 production was studied in freshly purified autologous B cells, cocultured with specifically activated T cells. The requirement of low Ag doses for *in vitro* induction of allergen-specific IgE was recently discussed [7]. As shown in Fig. 5(B), there was no difference in specific Ig production after stimulating the cells with either rPLA-WT or rPLA-H34Q.

Discussion

The aim of this study was to investigate whether the phospholipase activity of PLA is involved in immunoregulatory properties. Previous reports have shown that PLA can trigger degranulation and histamine release from the peritoneal connective tissue of rats and mice [38]. Preincubation of the enzyme with a specific inhibitor significantly suppressed PLA-mediated degranulation. It was also reported that IL-4 production is triggered by enzymatically active PLA in non-sensitized rat basophilic leukaemia cells, but not by enzymatically inactive PLA [40]. However, PLA does not induce histamine release from basophils of bee venom non-allergic patients [39]. The recombinant production of enzymatically active PLA and the H34Q point-mutated variant provide the possibility to study the effect of enzymatic activity on the quality of immune response.

Specific B cells present antigen most efficiently at low concentrations [7,49]. In spite of the low frequency of specific B cells, correctly folded PLA can bind directly to non-PLA-specific B cells, due to its high affinity for lipids (K^a : 10^{-13} M). Thus, non-specific B cells are able to present PLA to T cells with the same potency as PLA-specific B cells [45]. This indicates that any cell expressing MHC class II molecules might act as APC for PLA molecules having the correct three-dimensional configuration. Since rPLA-H34Q is recognized and presented by B cells in the same manner as rPLA-WT, the enzymatic activity of PLA may have no effect on the uptake of the antigen. Therefore, the binding capacity of PLA, which also depends on the correct



structure of the enzyme [50], is responsible for differences in immunological effects, rather than the enzymatic activity. This was confirmed by Moola *et al.* [51], who showed that L-asparaginase from *Erwinia chrysanthemi* bound 8-fold less wild-type specific Abs, after site-directed mutagenesis (P285T), but had equal enzymatic activity.

Exogenous antigens are taken up by APC, cleaved in endosomal compartments, and resulting peptides are presented to T cells in complex with the MHC class II molecule. Three linear T cell epitopes [29] and one carbohydrate dependent, HLA-DR restricted epitope [52] were identified on the PLA molecule. The catalytically important histidine at position 34 in PLA is not part of any of these epitopes. We showed that T cell proliferation did not change after stimulation with enzymatically inactive antigen compared to its active form. In addition, the cytokine pattern produced by rPLA-stimulated T cells was similar to the one induced by the enzymatically inactive analogue. This indicates that neither the site-mutation at position H34, nor the enzymatic PLA activity, influenced T cell triggering for both Th₁ and Th₂ cytokines.

Allergy to bee sting is largely related to excessive IgE Abs production against the PLA [26,29–31]. In contrast, development of IgG4 Abs against this molecule reflects normal immunity in exposed individuals [2,29]. However, the absolute level of one or the other isotype does not necessarily account for allergic or non-allergic responses. Of more relevance is the ratio of specific IgE:IgG4 Abs [29–31]. In these studies we show, that neither the absolute amounts nor the ratio was altered by stimulating T cells with PLA, lacking enzymatic activity but still exhibiting the correctly folded structure. Accordingly, modifications of the PLA molecule, which affect only the enzymatic activity, do not alter the allergic response of an individual.

In conclusion, our results demonstrate that the enzymatic activity of PLA has no effect on APC recognition, T cell proliferation, cytokine pattern or Ig production. This further indicates that any MHC class II expressing cell may act as APC for PLA, provided that the antigen adopts the correct three dimensional configuration. The reason for this is that only the intact structure of PLA is able to bind to the phospholipid enzymatic substrates, which are universal

constituents of the cellular membranes, by a K_d of $<10^{-13}$ M [53].

Therefore, the conformation of an allergen appears to be more important than the catalytic activity for MHC-expressing cells may act as APC for PLA. This knowledge excludes that PLA enzymatic activity influences T cell and B cell responses. Furthermore, it implicates that inactive PLA can be used in immunotherapy to avoid toxic side-effects during treatment.

Acknowledgements

The authors thank Dr Christoph H. Heusser and Dr Sefik S. Alkan, Novartis AG, Switzerland and Dr Jan DeVries,

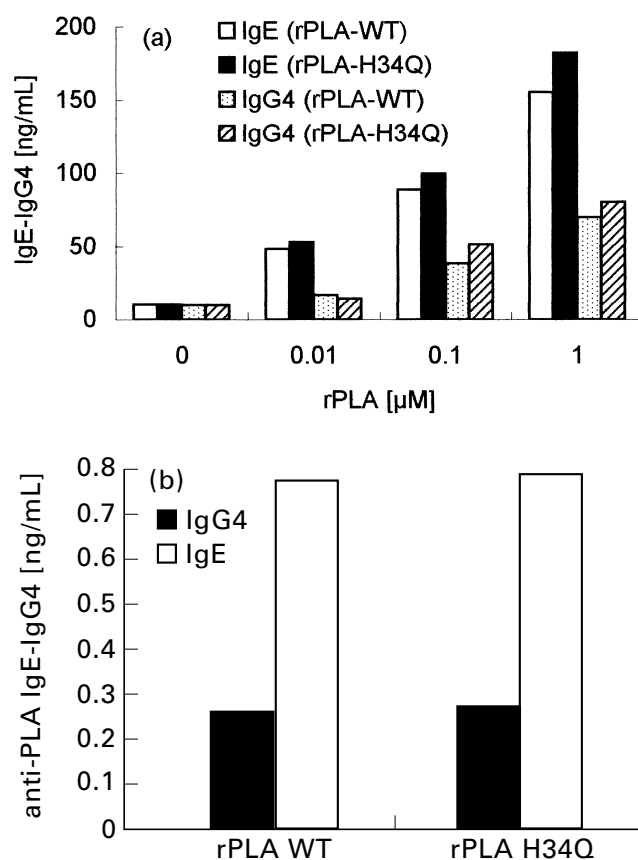


Fig. 5. Determination of total and PLA-specific IgE and IgG4. A, PBMC were stimulated with 0, 0.01, 0.1, and 1 μM of rPLA-WT or rPLA-H34Q, in the presence of IL-4 and sCD40L. Similar amounts of IgE and IgG4 were measured in rPLA-WT and rPLA-H34Q stimulated cultures after 12 days. B, PBMC were specifically stimulated and expanded with IL-2 and IL-4. After 12 days, cells were washed and reconstituted with freshly purified autologous B cells, and stimulated with 0.6×10^{-4} μM of the same PLA preparation. Supernatants were harvested after 12 days for determination of IgE anti-PLA and IgG4 anti-PLA Abs. The enzymatic activity had no effect on production and release of PLA-specific IgE and IgG4.

Fig. 4. Cytokine response to enzymatically active rPLA-WT or inactive rPLA-H34Q. PBMC of 11 allergic patients (solid lines) and three hyperimmune individuals (dashed lines) were stimulated with 1 μM of rPLA. Cultures were supplemented with IL-2 and IL-4 at day 7 and expanded when necessary. Cells were washed and restimulated with the same antigen, in the presence of autologous irradiated PBMC, at day 12. Supernatants were harvested 16 h later for IL-2 and IL-4, and 48 h later for IL-5, IL-13 and IFN-γ. No significant differences in cytokine production were observed. Background cytokine production in each patient was ≤ 20%.

DNAX Research Institute, CA for providing different cells and reagents.

This work was supported by the Swiss National Foundation, Grant no. 31.39.177.93, and the Theodor and Ida Herzog-Egli Foundation.

References

- Aalberse RC, van der Gaag R, van Leuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunisation results in an IgG4-restricted response. *J Immunol* 1983; 130:722–6.
- Aalberse RC, van Milligen F, Tan KY, Stapel SO. Allergen-specific IgG4 in atopic disease. *Allergy* 1993; 48:559–69.
- Romagnani S. Regulation of the development of type-2 T-helper cells in allergy. *Curr Opin Immunol* 1994; 6:838–46.
- Punnonen J, Aversa G, Cocks BG et al. Interleukin-13 induces interleukin-4 independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993; 90:3730–4.
- Pène J, Rousset f, Brière F, et al. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons- γ and - α and prostaglandin E_2 . *Proc Natl Acad Sci USA* 1988; 85:6880–4.
- Carballido JM, Carballido-Perrig N, Oberli-Schrämmli A, Heusser CH, Blaser K. Regulation of IgE and IgG4 responses by allergen-specific T cell clones to bee venom phospholipase A_2 in vitro. *J Allergy Clin Immunol* 1994; 93:758–67.
- Akdis CA, Blesken T, Akdis M et al. Induction and differential regulation of bee venom phospholipase A_2 -specific human IgE and IgG4 antibodies in vitro requires allergen-specific and nonspecific activation of T and B cells. *J Allergy Clin Immunol* 1997; 99:345–53.
- Zweiman B, Green G, Maycock R, Hildreth E. Inhalation sensitization to trypsin. *J Allergy* 1967; 39:11–6.
- Pepys J, Hargreave F, Longbottom L, Faux J. Allergic reaction of the lungs to enzyme of *Bacillus subtilis*. *Lancet* 1969; i:1181–4.
- Baur X, Fruhmman G. Allergic reaction, including asthma to the pineapple protease bromelain following occupational exposure. *Clin Allergy* 1979; 9:443–50.
- Baur X, König G, Bencze K, Fruhmman G. Clinical symptoms and results of skin test, RAST and bronchial provocation test in thirty three papain workers: evidence for strong immunogenic potency and clinically relevant proteolytic effects of airborne papain. *Clin Allergy* 1982; 12:9–17.
- Cartier H, Malo J, Pineau L, Dolowich J. Occupational asthma due to pepsin. *J Allergy Clin Immunol* 1984; 73:574–7.
- Baur X, Fruhmman G, Haug B, Rasche B, Reiher W, Weiss W. Role of *Aspergillus* amylase in bakers asthma. *Lancet* 1986; i:43.
- Tarvainen K, Kanerva L, Tupasela O et al. Allergy from cellulase and xylanase enzymes. *Clin Exp Allergy* 1991; 21:609–15.
- Müller UR. Insektenstichallergie: Klinik, Diagnostik und Therapie. Stuttgart: Gustav Fischer Verlag, 1988.
- Chua K, Stewart G, Thomas W, Simpson R, Dilworth R, Plozza T, Turner K. Sequence analysis of cDNA coding for a major house dust mite allergen, Der p I. Homology with cysteine proteases. *J Exp Med* 1988; 167:175–82.
- Lake F, Ward L, Simpson R, Thompson P, Stewart G. Allergenicity and physicochemical characterisation of house dust mite derived amylase. *Int Arch Allergy Appl Immunol* 1991; 94:357–8.
- Stewart G, Bird C, Thompson P. Do the group II dust mite allergens correspond to lysosyme? *J Allergy Clin Immunol* 1992; 90:141–2.
- Stewart G, Ward L, Simpson R, Thompson P. The group III allergen from the house dust mite *Dermatophagoides pteronyssinus* is a trypsin-like enzyme. *Immunology* 1992; 75:29–35.
- Thomas W. Mite allergens groups I–VII. A catalogue enzymes. *Clin Exp Allergy* 1993; 23:350–3.
- Novey HS, Marchioli LE, Sokol WN, Wells ID. Papain-induced asthma – physiological and immunological features. *J Allergy Clin Immunol* 1979; 63:98–103.
- Crameri R, Faith A, Hemmann S, Jaussi R, Ismail C, Menz G, Blaser K. Humoral and cell-mediated autoimmunity in allergy to *Aspergillus fumigatus*. *J Exp Med* 1996; 184:265–70.
- Hemmann S, Blaser K, Crameri R. Allergens of *Aspergillus fumigatus* and *Candida boidinii* share IgE-binding epitopes. *Am J Resp Crit Care Med* 1997; 156:1956–62.
- Robinson BWS, Venaille TJ, Mendis AHW, McAleer R. Allergens as proteases: An *Aspergillus fumigatus* proteinase directly induces human epithelial cell detachment. *J Allergy Clin Immunol* 1990; 86:726–31.
- Akiyama K, Shida T, Yasueda H et al. Allergenicity of acid protease secreted by *Candida albicans*. *Allergy* 1996; 51:887–92.
- Sabotka A, Franklin RM, Adkinson NFJ, Valentine M, Bauer H, Lichtenstein LM. Allergy to insect sting II. Phospholipase A. the major allergen in honey bee venom. *J Allergy Clin Immunol* 1976; 57:29–40.
- Müller UR, Dudler T, Schneider T et al. Type 1 skin reactivity to native and recombinant phospholipase A_2 from honey bee venom is similar. *J Allergy Clin Immunol* 1995; 96:395–402.
- Schneider T, Lang AB, Carballido JM et al. Human monoclonal or polyclonal antibodies recognize predominantly discontinuous epitopes on bee venom phospholipase A_2 . *J Allergy Clin Immunol* 1994; 94:61–70.
- Carballido JM, Carballido-Perrig N, Kägi MK et al. T cell epitope specificity in human allergic and nonallergic subjects to bee venom phospholipase A_2 . *J Immunol* 1993; 150:3582–91.
- Akdis AC, Akdis M, Blesken T, Wymann D, Alkan SS, Blaser K. Epitope specific T cell tolerance to phospholipase A_2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. *J Clin Invest* 1996; 98:1676–83.
- Wyss M, Scheitlin T, Stadler BM, Wüthrich B. Immunotherapy with aluminium hydroxide adsorbed insect venom extracts (Alutard SQ): immunologic and clinical results of a prospective study over 3 years. *Allergy* 1993; 48:81–6.
- Kuchler K, Gmachl M, Sippl MJ, Kreil G. Analysis of the cDNA for phospholipase A_2 from honey bee venom glands. *Eur J Biochem* 1989; 184:249–54.

- 33 Scott DL, Otwinowski Z, Gelb MH, Sigler PB. Crystal structure of bee-venom phospholipase A₂ in a complex with a transition-state analogue. *Science* 1990; 250:1563–6.
- 34 Scott DL, White SP, Otwinowski Z, Yuan W, Gelb MH, Sigler PB. Interfacial catalysis: The mechanism of phospholipase A₂. *Science* 1990; 250:1541–6.
- 35 van den Bosch H. Intracellular phospholipases A. *Biochim Et Biophys Acta* 1980; 604:191–246.
- 36 Pruzanski W, Vadas P. Phospholipase A₂ – a mediator between proximal and distal effectors of inflammation. *Immunol Today* 1991; 12:143–6.
- 37 Chiu HF, Chen IJ, Teng CM. Edema formation and degranulation of mast cells by a basic phospholipase A₂ purified from *Trimeresurus mucrosquamatus* snake venom. *Toxicon* 1989; 27:115–25.
- 38 Murakami M, Hara N, Kudo I, Keizo I. Triggering of degranulation in mast cells by exogenous type II phospholipase A₂. *J Immunol* 1993; 151:5675–84.
- 39 Förster E, Dudler T, Gmachl M, Aberer W, Urbanek R, Suter M. Natural and recombinant enzymatically active or inactive bee venom phospholipase A₂ has the same potency to release histamine from basophils in patients with hymenoptera allergy. *J Allergy Clin Immunol* 1995; 95:1229–35.
- 40 Dudler T, Cantarelli Machado D, Kolbe L et al. A link between catalytic activity, IgE-independent mast cell activation and allergenicity of bee venom phospholipase A₂. *J Immunol* 1995; 155:2605–13.
- 41 Mueller HL. Diagnosis and treatment of insect sensitivity. *J Asthma Res* 1966; 3:331–3.
- 42 Lane P, Bocker T, Hubele S, Padovan E, Lanzavecchia A, McConnell FF. Soluble CD40 ligand can replace the normal T cell derived CD40 ligand signal to B cells in T cell-dependent activation. *J Exp Med* 1993; 177:1209–13.
- 43 Dudler T, Chen W, Wang S et al. High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A₂. *Biochim Et Biophys Acta* 1992; 1165:201–10.
- 44 Thannhauser TW, Konishi Y, Scheraga HA. Sensitive quantitative analysis of disulfide bonds in polypeptides and proteins. *Anal Biochem* 1984; 138:181–8.
- 45 Carballido JM, Faith A, Carballido-Perrig N, Blaser K. The intensity of T cell receptor engagement determines the cytokine pattern of human allergen-specific T helper cells. *Eur J Immunol* 1997; 27:515–21.
- 46 Holmes K, Fowlkes BJ, Schmid I, Giorgi JV. Preparation of cells and reagents for flow cytometry. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds *Current protocols in immunology*. (Suppl. 16) Edn. New York: John Wiley & Sons, Inc. 1995:5.3.5–6.
- 47 Yuan W, Quinn DM, Sigler PB, Gelb MH. Kinetic and inhibition studies of Phospholipase A₂ with short-chain substrates and inhibitors. *Biochemistry* 1990; 29:6082–94.
- 48 Gillis S, Ferm WW, Ou W, Smith KA. T cell growth factor: parameters of production and a quantitative microassay for activity. *J Immunol* 1978; 120:2027–3032.
- 49 Secrist H, DeKruyff RH, Umetsu DT. Interleukin 4 production by CD4⁺ T cells from allergic individuals is modulated by antigen concentration and antigen presenting cell type. *J Exp Med* 1995; 181:1081–9.
- 50 Akdis AC, Blesken T, Wymann D, Akdis M, Faith A, Blaser K. Allergen conformation-dependent T cell cytokine and antibody isotype patterns in bee sting sensitized subjects. *J Allergy Clin Immunol* 1996; 97:246.
- 51 Moola ZB, Scawen MD, Atkinson T, Nicholls DJ. *Erwinia chrysanthemi* l-asparaginase: epitope mapping and production of antigenically modified enzymes. *Biochem J* 1994; 302:921–7.
- 52 Dudler T, Altmann F, Carballido JM, Blaser K. Carbohydrate-dependent, HLA class II-restricted, human T cell response to the bee venom allergen phospholipase A₂ in allergic patients. *Eur J Immunol* 1995; 25:538–42.
- 53 Ramirez F, Jain MK. Phospholipase A₂ at the bilayer interface. *Proteins* 1991; 9:229–39.